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SESSION E. USES AND CAMELLIA PRODUCTS

Bioactive compounds and biological properties of oils from three *Camellia* species

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Abstract. The concentration of polyphenols and antimicrobial and antioxidant properties of oils obtained from seeds of *Camellia japonica, Camellia sasanqua* and *Camellia grijsii*, grown in Pontevedra (NW Spain), were studied. Oils were obtained by cold-pressed extraction. Standard methods for moisture, density and acid and iodine values were used to analyse the stability of the fresh oils. Strains of *Klebsiella pneumonia*, *Salmonella* sp., *Staphylococcus epidermidis* and *Cryptococcus neoformans*, all infectious pathogens in humans, were used to assess the antimicrobial properties of the oils.

The concentration of polyphenols ranged from 0.02± 0.006 to 0.04 ± 0.001 mg of gallic acid per g in oils from *C. japonica* and *C. grijsii*, respectively. Concerning the antioxidant activity, determined using the DPPH• scavenging activity and the β-carotene bleaching assay. The highest value was found in *C. grijsii* and the lowest in *C. japonica*.

All the oils showed antimicrobial activity, and exhibited different selectivity and Minimum Inhibitory Concentrations (MIC) for each microorganism under study. The most sensitive was *K. pneumonia*, followed by *Salmonella* sp., *S. epidermidis* and *C. neoformans*, the highest MIC value being found for *C. neoformans*. For all the microorganisms, the higher antimicrobial activity was obtained from the oil of *C. grijsii*, followed by C. *sasanqua* and *C. japonica.*

Since the highest biological properties were found in the oil containing the highest concentration of polyphenols, it can be hypothesised that these bioactive compounds might play a role in those properties.

Keywords: antimicrobial activity, antioxidant activity, phenolic compounds.

Introduction

Camellia oil has been traditionally used for cooking and as a protective cosmetic to maintain the health of skin and hair in Asian cultures (Jung et *al*., 2007). Moreover, camellia oil is often the target for adulteration or mislabelling because it is a high priced product with high nutritional and medical values (Wang et *al*., 2006).

Camellia oil has been recognized as a source of vitamins A, B and E, essential fatty acids, primarily as oleic acid, and also polyunsaturated fatty acids such as omega-6 linoleic acid (Li *et al.*, 2012). In a previous work, Salinero et al. (Salinero *et al.*, 2011) have characterized the triglyceride composition of oil from *C. reticulata* and *C. japonica* harvested in Galicia (North Western Spain) by nuclear magnetic resonance (NMR). Furthermore, other functional components, such as camellia saponins, squalene or polyphenolic compounds have been found in camellia oil. All these compounds appear to be very promising for possible pharmaceutical exploitation since they confer anti-tumor effects, blood lipid reduction, protection of liver and heart, antisepsis and anti-inflammation effects, atherosclerosis delaying, anti-oxidation activities and immune function regulation on the population (He *et al.*, 2011).

Even though *Camellia* spp. have been used in ethnomedicine, there is relatively little information regarding the biological activity of *Camellia* species' oils, except for tea oil (Feás *et al*., 2013).

This work investigates and establish for the first time, the physicochemical characterization, bioactive compounds, antimicrobial and antioxidant properties of Galician camellia oils from 8 different *Camellia japonica* cultivars, *1 C. sasanqua* cultivar and *1 C. grijsii*.

Materials and Methods

All the chemicals and solvents used were of analytical grade.

Camellia seed oils

Seeds from 10 different camellia specimens, 8 *Camellia japonica* cultivars, 1 *C. sasanqua* cultivar and 1 *C. grijsii*, were harvested in the Galician region (North-western Spain). Table 1 shows the label, camellia specie and origin of the samples under study.

Table 1. Sample name, camellia specie and location of the samples under study

Sample name	*Specie*	Location
1-L	*C. japonica*	Pazo de Lourizán, Pontevedra
1-O	*C. japonica*	Pazo de Oca, A Estrada
16-A	*C. japonica*	EFAreeiro, Pontevedra
19-S	*C. japonica*	Soutomaior Castle, Soutomaior
33-G	*C. japonica*	Pazo de Gandarón, Pontevedra
71-S	*C. japonica*	Soutomaior Castle, Soutomaior
198-A	*C. sasanqua*	EFAreeiro, Pontevedra
224-A	*C. grijsii*	EFAreeiro, Pontevedra
284-A	*C. japonica*	EFAreeiro, Pontevedra
531-R	*C. japonica*	Pazo de Rubiáns, Vilagarcía

The seeds were cleaned by hand to remove the foreign materials and then washed with tap water. Cleaned seeds were dried at room temperature. After these treatments, the seeds were ground in a mill. Camellia oil samples were obtained by cold-pressed extraction. About 200 g of crushed seeds are loaded into a hydraulic press and then, they are pressed by mechanical means to extract the oil. The crude oil portions belonging to a given *camellia* cultivar were blended and decanted and filtered. Finally, the oils were stored in amber coloured glass bottles and kept at 15 °C until further analysis.

Chemical and physical methods

Moisture and volatile matter determination

Moisture and volatile matter was determined gravimetrically according to the UNE-EN ISO 662-1998 standard method (ISO 662-1998, 2001) as the mass loss suffered by five millilitres of oil sample after heating in an oven at 103 ± 2 °C. Determinations were performed in duplicate. Results were expressed as mass percentage.

Density determination

The density of the samples, expressed as the average value of six replicates, was determined as the measure of the oil mass per unit volume at 25 °C.

Acid value

The acid value (AV) was determined by a standard titration procedure according to the UNE-EN ISO 660:2009 (ISO 660:2010, 2010). An amount of 10 g of camellia oil was dissolved in a 50:50 mixture of ethanol (purity 96%) and diethyl ether (purity 99.9%). Finally, the acids in solution were titrated with 0.1 M KOH prepared in ethanol solution. Results were expressed as grams of oleic acid per 100 g of oil. These determinations were carried out in triplicate.

Iodine value

The iodine value (IV) was calculated according to the standard method UNE-EN ISO 3961:2009 (ISO 3961:2009, 2012). Data were obtained from the analysis of each sample oils prepared in triplicate. About 0.20 g of sample oil was dissolved in a mixture of cyclohexane and glacial acetic acid (50:50). Finally, the excess of iodine generated after adding Wijs solution, potassium iodide and deionized water, was titrated with sodium thiosulphate. Results were expressed as grams of iodine per 100 grams of oil.

Peroxide value

To determine the peroxide value (PV), a portable HANNA instruments® photometer HI 83730 (Guipúzcoa, Spain) equipped with a commercially available kit of reactive was used. Results, expressed as milliequivalents of oxygen per kilogram of oil, were calculated as the average value of three replicates of the same sample.

Spectrophotometric determination of polyphenols

The determination of the polyphenols was carried out according to Capannesi et al. (2000). Galic acid standard solutions using methanol were used for constructing the calibration curve ($y = 0.3442x + 0.0062$; $R^2 = 0.9977$).

It was prepared a 50 mL solution containing: 1 ml of a standard solution of gallic acid, 6 ml of methanol, 2.5 mL of the Folin±Ciocalteau reagent and 5 ml of 7.5% Na_2CO_3. The solutions were stored overnight and the spectrophotometric analysis was performed at λ=765 nm.

The determination was carried out by diluting 2.5 g of camelia oil in 2.5 mL of n-hexane. Then, the mixture was extracted three times by 5 min centrifugation (5000 rpm) with CH_3OH:H_2O (80:20 v/v). The extract was added to 2.5 mL Folin±Ciocalteau reagent, 5 mL of Na_2CO_3 (7.5%), in a 50 mL volumetric flask to which purified water was added. The samples were stored overnight, and the spectrophotometric analysis was performed at λ=765 nm.

DPPH Scavenging Activity

Various concentrations of oil extract (300 µL) were mixed with 2.7 mL of MeOH solution containing DPPH•, at a concentration of 6×10^{-5} mol/L. The mixtures were shaken vigorously and left 60 min in the dark, until stable absorption values were obtained. The reduction of the DPPH• was measured by continuous monitoring the decrease of absorption at 517 nm. The radical-scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation: $\%RSA = [(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, being A_S the absorbance of the solution when the extract oil was added at a particular level and A_{DPPH} the absorbance of the DPPH solution (Morais et al., 2011).

β-Carotene Bleaching (BCB) Assay

This assay has been previously described by Feás et al. (2013). In brief, 2 ml of β-carotene solution (2 mg β-carotene/10 mL chloroform) were added to 40 mg of linoleic acid and 400 mg of Tween 20. The mixture was shaken and portions of 4.8 mL were transferred into different test tubes containing different oil concentrations (200 µL). After that, these mixtures were incubated in a water bath at 50 °C for 60 min. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm. Absorbance readings were then recorded at 20-min intervals until it was observed changes in the colour of the control sample. A blank, in the absence of β-carotene, was prepared for background subtraction. Lipid peroxidation inhibition (LPO) was calculated using the equation: LPO = (β-carotene content after 2 h of assay/initial β-carotene content) × 100.

Microbial Strains

In the present study it was used microorganisms isolated from biological fluids, collected in the Northeast Hospital Centre (Bragança, Portugal) and identified in the Microbiology Laboratory of Escola Superior Agrária de Bragança, using molecular biology techniques.

The isolates were stored in Muller-Hinton medium with 20% glycerol at -70ºC. The inoculum for the assays were prepared by diluting cell mass in 0.85% NaCl solution adjusted to 0.5 MacFarland scale confirmed spectrophotometricaly at 580 nm for bacteria and 640 nm for yeasts. Cell suspensions were finally diluted to 10^4 CFU/mL.

Determination of the antimicrobial activity

Antimicrobial activity was carried out according to Morais et al. (2011), using Nutrient Broth (NB) or Yeasts Peptone Dextrose (YPD) on microplate (96 wells).

The extracts were_diluted in dimethylsulfoxide (DMSO) and transferred into the first well and serial_dilutions were performed. The inoculum was added to all wells and the plates were incubated at 37ºC. Fluconazol and gentamicine were used as controls. It was used a positive control (inoculated medium), a negative control (medium) and a DMSO control (DMSO with inoculated medium). Antimicrobial activity was detected by adding 20 µL of 0.5% TTC solution. The Minimum Inhibitory Concentration (MIC) was considered to be the lowest concentration of the tested sample able to inhibit the growth of bacteria after 24 h and fungi after 48 h, as indicated by the TCC staining (dead cells are not stained by TTC). All the tests were performed in triplicate (n = 3) and the results are expressed as mg/mL.

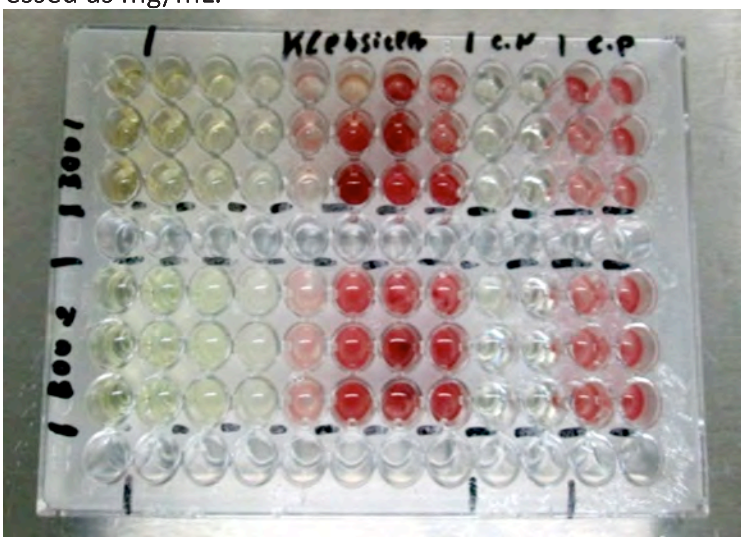

Fig. 1Determination of the antimicrobial activity

Statistical analysis

Results are shown as mean values ± standard deviation. In each parameter the differences between the samples were analysed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Post-hoc test, in which p≤0.05 were considered significant.

Results and Discussion

The percentage of extraction of the Camellia grijsii (7.93±1.48%) was significantly lower than the obtained for the other species. The density of the three types of oils was equal to 0.90g/mL. The percentage of moisture of the *Camellia japonica*'s oil (0.23±0.13%) differed significantly from the determined for the oil of *Camellia sasanqua* (0.03±0.02%) and *Camellia grijsii* (0.04±0.03%). Significant differences were also obtained regarding the iodine index, which ranged from 80.06±0.59 (*C. grijsii*) to 85.66±0.26 g/100g (C. sasanqua). Concerning the acidity and peroxide index, no significant differences were obtained between the three types of oil under study. As it can be seen, Table 2 shows the physical characteristics of the oils.

Table 2. Physical characteristics of the oils. For a given *physical parameter,* mean values with the same letters (a or b) were similar and no statistically significant differences were observed between samples (p<0.05)

Physical parameters	*Camellia japonica*	*Camellia sasanqua*	*Camellia grijsii*
Extraction (%)	22.35±3.85 b	24.28±5.06 b	7.93±1.48 a
Density (g/mL)	0.90±0.01 a	0.90±0.03 a	0.90±0.02 a
Moisture (%)	0.23±0.13 b	0.03±0.02 a	0.04±0.03 a
Iodine Index (g/100g)	81.39±1.88 a	85.66±0.26 b	80.06±0.59 a
Acidity Index (g/100g)	0.92±0.72 a	0.49±0.04 a	0.03±0.03 a
Peroxide Index (meqO_2/kg)	1.16±0.84 a	0.63±0.73 a	0.38±0.33 a

Regarding the antimicrobial activity, all the oils efficiently inhibited the microorganisms under study. The most sensitive specie was *Klebsiella* ESA53, especially when *Camellia grijsii* oil was used, being the minimum inhibitory concentration equal to 5.11±2.05 mg/mL. The minimum inhibitory concentration of the three oils against *Salmonella* varied between 10.84±4.06 mg/mL (*C. grijsii*) and 12.91±4.18 (*C. japonica*). Regarding *S. epidermides* the most efficient oil was, again, the obtained from *Camellia grijsii* and the least efficient was the extracted from *C. sasanqua*. *Cryptococcus neoformans* was the most resistant microorganism and the three minimum inhibitory concentrations did not differ significantly (p><0.05). Table 3 shows the antimicrobial activity of the three types of oil under study

Table 3. Antimicrobial activity of the three types of *Camellia* spp. oil. For a given *specie,* mean values with the same letters (a or b) were similar and no statistically significant differences were observed between samples (p<0.05)

Specie	***Klebsiella* ESA53(mg/mL)**	***Salmonella* ESA26(mg/mL)**	***S.epidermides* ESA143 (mg/mL)**	***C. neoformans* ESA125 (mg/mL)**
Camellia japonica	6.36±1.49 b	12.91±4.18 a	13.67±5.52 a	25.53±7.39 a
Camellia sasanqua	5.76±1.98 b	12.19±4.72 a	13.12±6.03 a	24.34±8.89 a
Camellia grijsii	5.11±2.05 a	10.84±4.06 a	11.21±5.06 a	22.32±8.95 a

Concerning the phenolic compounds the concentrations were between 0.02±0.001 and 0.04±0.00, for *Camellia japonica* and for both *C. sasanqua* and *C. grijsii,* respectively. Antioxidant activity was higher in *C. grijsii*, independently of the used methodology. Results are shown in Table 4. Significant differences were observed for the bioactive compounds and antioxidant activity between the oils. In addition, the species with higher concentration of polyphenols were the most efficient in the neutralization of free radicals.

Table 4. Polyphenolic compounds and antioxidant activities of the *Camellia* spp. oils. For a given assay, mean values with the same letters (a or b) were similar and no statistically significant differences were observed between samples ($p<0.05$)

Specie	Polyphenols (mg gallic acid/g)	DPPH scavenging (mg/mL)	LPO inhibition (mg/mL)
Camellia japonica	0.02±0.01 a	58.03±7.07 b	0.85±0.07 b
Camellia sasanqua	0.04±0.00 b	48.31±3.61 ab	0.77±0.01 ab
Camellia grijsii	0.04±0.00 b	39.06±4.64 a	0.69±0.03 a

Conclusions

The obtained results demonstrate the antioxidant and antimicrobial activities of the three *Camellia* spp. oils, being *Klebsiella* the most sensitive to the inhibitory effect.

The finding hereby reported open new possibilities for future therapeutic applications of the *Camellia* oils, even though further studies are needed to analyse the potentialities of the *in vivo* use.

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